DERIVATIZED AGAROSE AND METHOD OF MAKING AND USING SAME

This invention relates to derivatized agarose, particularly to derivatized agarose having new and novel uses in electrophoresis and diffusive interactions.

In the electrophoresis art it is known to use sieving gels for the separation of biological substances. Such gels are characterized by the presence of a microporous structure which exerts a selective action on the migrating fractions, restricting passage of the high molecular weight fractions while permitting passage of the low molecular weight fractions. The technique was originally carried out using starch as the sieving gel; Smithies, O., Biochem, J. 61,629 (1955). However, starch gels suffers from several disadvantages; 1. they are not clear; 2. they are discontinuous and therefore cannot be dried and preserved intact; 3. their dimensional strength is low; 4. their range of porosities is limited; 5. proteins separated in them are recovered in poor yields and as recovered contain low M.W. starch contaminants. Later, polyacrylamide was developed as an electrophoretic gel sieving medium. A "stacking gel" variation was subsequently devised and this led to disc electrophoresis as a popular gel sieving tool.

Polyacrylamide forms exceptionally clear, typically continuous gels which can be dried and stored without fracturing. They can be prepared in a wide range of concentrations and consequent porosities.

Although an advance over the earlier starch gels, polyacrylamide gels were not entirely satisfactory and exhibited several major disadvantages. For instance, the acrylamide monomer used to form the polyacrylamide is a cumulative neurotoxin and varying amounts remain in the polymerized gels depending on the conditions of polymerization thereby creating a health hazard. The conditions of polymerization can also affect gel porosity and the tendency of these gels to swell differently in different aqueous solutions. This has limited the reproducibility of these gels and thus curtailed their widespread use. Moreover, polyacrylamide gels notoriously have low gel strength, particularly at low concentrations. Furthermore, such gels are difficult to destain and use as a preparative gel medium because of their retentiveness and lack of freeze-thaw characteristics.

The many advantages and uses of sieving gels has provided a strong incentive to develop new and improved types. Although numerous publications have appeared on this subject, none have described convenient methods which are nondenaturing to the proteins separated and are also amenable to the range of conditions (urea, SDS, triton, etc.) which are commonly used in such separation techniques.

To overcome the dimensional strength liability of low concentration polyacrylamide gels, it has been proposed to incorporate agarose into the gel; Peacock, A. et al., Biochemistry 7,668 (1968). A similar approach was taken by Bode, Anal. Biochemistry 83,204 (1977) who incorporated liquid polyacrylamide into agar gels but found that the practical upper limit for such formulations was only 5%. In Anal. Biochemistry 70,32 (1976) by Chrambach, A. et al., there is disclosed the use of diallyltartardiimide (DATD) instead of disacrylamide as a means of increasing adhesion and therefore mechanical stability of the polyacrylamide gels in glass tubes. This system also facilitates resolubilization of the gels by oxidative hydrolysis although it tends to degrade proteins.

Similarly, attempts to recover proteins separated in polyacrylamide by swelling the gel with quaternary ammonium compounds or dissolving them in H_2O_2 also risk danger to the proteins and the researcher isolating them; Peacock, A. and Dingman, C., Biochemistry 7,668 (1968).

As a means of overcoming the difficult protein recovery characteristics of polyacrylamide gel for preparative applications, there is described by Brown, M. et al., Electrophoresis '79, page 235, Ed. B. Radola, Walter DeGruyter, Berlin (1980) a method of cross-linking which could be reversed by treatment with 1 M NaOH. However, such harsh conditions risked destroying the very protein samples which were being analyzed.

The attempts to combine agarose and polyacrylamide, Brown, M., et al., Electrophoresis '79, page 235, Ed. B. Radola, Walter DeGruyter, Berlin (1980) and Lowe, C., Int. J. Biochemistry, 8,177 (1977) were based on the recognition that these two gel media had almost opposite characteristics: whereas agarose has a high gel strength at low concentrations, polyacrylamide has a low gel strength at low $\leqq 7\%$ concentrations. Whereas agarose gels typically exhibit a slight translucence, polyacrylamide gels in many formulations are crystal clear; whereas agarose exhibits very little sieving toward most serum proteins 50,000–500,000 MW polyacrylamide gels are the medium of choice for proteins within this range of molecular weights, Sargent, J. R. in "Methods in Zone Electrophoresis," page 75, pub by BDH Ltd, Poole, England, 2nd Ed. (1969). In practice, however, the composite gel approach met with only limited success. The traditional application of these two media employs agarose for the sieving separation of proteins $>500{,}000$ MW. While those $<500{,}000$ (MW) daltons are best resolved in polyacrylamide gels which can readily be formed so as to have exclusion limits $<2{,}000$ (MW) daltons, Ashton, G., Nature, 180,917 (1957) and BioRad 1980 Catalog, Page 36, BioRad Laboratories, Richmond, Calif. The approach of "filling" with polyacrylamide the large, highly porous agarose gels whose ultrastructure has been reported in detail by Rees, et al., J. Molecular Biology 90,269 (1974) therefore ultimately proved to be a mediocre remedy and compromised some of the best properties of each medium.

Another approach to the development of sieving media for both electrophoretic and chromatographic applications has been to cross-link otherwise nongelling, neutral polysaccharides under appropriate concentration conditions. Examples of such cross-linked sieving media are: locust bean gum, Duel, H. and Neukon, H., Advan. Chem. Ser., No. 11,51 (1954) Dextran, Flodin, P., Dextran gels and their applications in gel filtration, Pharmacia, Uppsala, Sweden (1962) Guar gum, Gupta, K., Sahni, M. et al., J. Chromatography 169,183 (1979), and cellulose, Peska, J. and Stamberg, J., U.S. Pat. No. 4,055,510 (Oct. 25, 1977). The principal disadvantages of such procedures are: that the gels cannot be readily dissolved after cross-linking for preparative protein recovery applications, and the gels cannot be readily polymerized in the presence of the necessary buffers or ampholytes required for most electrophoretic separation techniques.

A modified agarose is described in U.S. Pat. No. 3,956,273 to Guiseley. The product is prepared by introducing certain substituents into the disaccharide mole-